Note

Plant Biotechnology, 21 (1), 73-77 (2004)

Molecular Cloning of a cDNA that Encodes a Spinach Cysteine Synthase-Like Protein

Ryosuke ASAI, Junko NAKATA, Kyoko TAKAHASHI, Hiroaki KODAMA* and Masahiro MASADA

Department of Bioproduction Science, Faculty of Horticulture, Chiba University, Yayoi-cho 1-33, Inage-ku, Chiba 263-8522, Japan

**Corresponding author* E-mail address: kodama@faculty.chiba-u.jp

Received 21 August 2003; accepted 10 October 2003

Abstract

In *Spinacia oleacea*, three isoforms of cysteine synthase (CSase, *O*-acetyl-serine (thiol) lyase) were identified in the cytosolic, plastidial, and mitochondorial compartments. Here, we report molecular cloning of a cDNA that encodes a CSase-like protein, designated CSaseLP. The predicted amino acid sequence of CSaseLP showed high identity (about 70%) with that of a spinach cytosolic CSase (encoded by *CSaseA* gene). The CSase activity of recombinant CSaseLP was barely detectable. The antibody raised against the recombinant CSaseLP protein recognized weakly a recombinant CSaseA protein. Northern blot analysis showed that *CSaseLP* gene was expressed mainly in root tissues, while the expression of *CSaseA* gene was constitutive in leaf and root tissues. These results suggest that the CSaseLP protein, though belongs to the CSase family, play a role distinct from the cytosolic CSase.

Accession number: AB113874.

Key words: cysteine synthase, pyridoxal phosphate, spinach.

Abbreviations

3′RACE, rapid amplification of 3′-cDNA ends; 5′RACE, rapid amplification of 5′-cDNA ends; CASase, cyanoalanine synthase; CSase, cysteine synthase; IPTG, isopropylthiogalactoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RT, reverse transcription; SDS, sodium dodecyl sulfate.

Higher plants and microorganisms reduce inorganic sulfate to cysteine by the sulfate assimilation pathway. The last step is catalyzed by cysteine synthase (CSase), which synthesizes L-cysteine from *O*-Acetyl-L-serine and sulfide. We have purified four different CSases from spinach leaves (Yamaguchi and Masada, 1995; Yamaguchi *et al.*, 1998). The cDNAs encoding CSase isozymes were also isolated from spinach. The *CSaseA, CSaseB* and *CSaseC* encode cytosol, chloroplast and mitochondrion-localized CSases, respectively (Saito *et al.*, 1992, 1993, 1994).

The isozymes of the CSase have the potential to synthesize several β-substituted alanines by reactions analogous to cysteine synthesis. For example, two cysteine synthases were purified from *Quisqualis indica*, and one of them can catalyze the synthesis of L-quisqualic acid [β-(3,5-dioxo-1,2,4-oxadiazolidin-2-yl)-L-alanine] (Murakoshi *et al.*, 1986). The CSases purified from spinach leaves showed the cyanoalanine synthase (CASase) activity (Hatzfeld *et al.*, 2000). In fact, an N-terminal amino acid sequence of a CASase purified from spinach is identical with the corresponding amino acid sequence of mitochondrial CSase isozyme (Warrilow and Hawkesford, 2000). In *Arabidopsis*, a mitochondrial CSase, AtcycC1 protein, showed CASase activity, but other two cytosolic CSases did not (Yamaguchi *et al.*, 2000). A potato mitochondrial CSase showed preferred substrate specificity for CASase rather than for CSase reaction (Maruyama *et al.*, 2000, 2001). These results suggest that determination of native *in vivo* function of the CSase isozymes is rather difficult from comparison of their primary amino acid sequences.

Among four kinds of spinach CSases purified in our laboratory, two CSases showed different amino acid sequences from those of the CSaseA, CSaseB or CSaseC (Yamaguchi *et al.*, 1998). In our study for the isolation of cDNAs of these CSases, we have amplified cDNA fragments by RT-PCR with several pairs of degenerate oligonucleotide primers. When a pair of degenerate primers synthesized based on the sequences of *CSaseA* cDNA and

CSaseB cDNA was used, we obtained a cDNA fragment which is somewhat different from any of the CSase cDNA. The full-length cDNA sequence of this cDNA was deteremined, which showed high similarity to the *CSaseA* cDNA. Since a recombinant protein produced using this cDNA clone showed quite limited CSase activity, it was designated CSase-like protein (*CSaseLP*) gene. In this report, the molecular cloning and characterization of the *CSaseLP* cDNA is described.

Isolation of total RNA from young fresh spinach leaves, purchased from a local market, was performed according to the procedure of Kodama *et al.* (1997). Single-stranded cDNAs were synthesized with Omniscript reverse transcriptase (QIAGEN). cDNA fragments encoding CSases were amplified with primers 5′-CC(C/T)AC(C/G)AG(C/T)GG(A/G)AATACTGG-3′ and 5′-A(A/G)(A/G)TATC(G/T)(C/T)TC(A/G)CCAAAGCT-3′, which were designed according to the *CSaseA* and *CSaseB* cDNA sequences. The products of polymerase chain reaction (PCR) were cloned into the pUC18 plasmid and sequenced. Rapid amplification of 5′-cDNA ends (5′RACE) was carried out using 5′RACE system version 2.0 (Gibco BRL), and the rapid amplification of 3′-cDNA ends (3′RACE) was performed with TaKaRa RNA LA PCR Kit version 1.1 (TaKaRa Biomedicals). The *CSaseLP* cDNA fragment with a complete open reading frame was obtained by reverse transcription (RT)-PCR with primers 5′-GTGTAGCTTTGCTTGGGTAT-3′ and 5′-GTCTCATTTTTCAGCGGGTG-3′. After determination of nucleotide sequence of the resultant cDNA, *Eco*RI and *Sal*I sites were added to the 5′ end and 3′ end of the *CSaseLP* cDNA, respectively. This was done by means of PCR with primers 5′-GAATTCATGGAGGGAGAGTGCA-GCAT-3′ and 5′-GTCGACTCACAAGGGG-GAAAGTGGCA-3′. Similarly, *Eco*RI and *Sal*I sites were added to the 5′ end and 3′ end of the *CSaseA* cDNA by means of PCR with primers 5′-GAATTCATGGTTGAGGAGAAGGCCTT-3′ and 5′-GTCGACTTAGGACTCAATAACCATGC-3′, respectively. These amplified *CSaseLP* and *CSaseA* cDNA fragments were subcloned into the *Eco*RI-*Sal*I sites of pET28 vector (Novagen) to give plasmids, pET-CS-LP and pET-CS-A, respectively.

The plasmids, pET-CS-LP and pET-CS-A, were introduced into *Escherichia coli* BL21-CodonPlus(DE3)-RP cells (Stratagene). Production of recombinant proteins was induced by addition of isopropylthiogalactoside (IPTG) at a final concentration of 1 mM. After 2 h culture, cells were harvested by centrifugation. The *E. coli* cells were suspended in 100 mM potassium phosphate (pH 8.0) buffer containing 8 M urea, and the mixture was sonicated. After centrifugation at 10,000*g* for 10 min, the supernatant was loaded onto a Hi-Trap chelating column (Pharmacia). The column was washed with 20 mM sodium phosphate buffer (pH 7.4) containing 8 M urea, 0.5 M NaCl and 10 mM imidazole, and the recombinant proteins were eluted with 20 mM sodium phosphate buffer (pH 7.4) containing 8 M urea, 0.5 M NaCl and 500 mM imidazole.

A single polypeptide for a recombinant CSaseLP protein was isolated using the eluted fraction from the Hi-Trap column by preparative sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis (PAGE). Rabbits were immunized with this purified recombinant protein (Tanpaku Seisei Kogyo, Japan). Proteins were separated by native PAGE or by SDS-PAGE as previously described (Yamaguchi *et al.*, 1998). After electrophoresis, proteins were electroblotted onto a polyvinylidene difluoride membrane. Western blotting was carried out as previously reported (Kitamura *et al.*, 1996). Solubilized proteins eluted with urea from the Hi-Trap column were folded by the slow removal of urea, which was preformed by a step-wise dialysis under the presence of 1 mM pyridoxal phosphate. The concentration of urea in the dialysis buffer gradually decreased. After removal of urea, the resultant solution was centrifuged and the supernatant was subjected to the CSase assay as previously described (Yamaguchi *et al.*, 1998).

The cDNA inserts of *CSaseA* and *CSaseLP* were subcloned into pGEM3Zf (Promega). The digoxygenin-labelled antisense RNA probe was prepared by the manufacturer's protocol. Hybridized probes were visualized using CDP-star reagent (Amersham Biotechnology).

The full-length cDNA sequence of the *CSaseLP* gene was determined by 5′ and 3′ RACE, and it harbored 996 bp open reading frame. As shown in **Fig. 1**, the amino acid sequence deduced from the *CSaseLP* cDNA showed a continuous stretch of high similarity with the CSase genes. The identity of the predicted amino acid sequence of *CSaseLP* was 69% to CSaseA, 60% to CSaseB, and 58% to CSaseC. The deduced molecular mass of CSaseLP protein is 35.7 kDa. In the amino terminal region of CSaseLP protein, there was no apparent extension with a characteristic feature of transit peptides of plastid or mitochondrial CSase isoforms, suggesting that the CSaseLP protein is localized in cytosol. The lysine residue, a binding site to the pyridoxal phosphate cofactor, and its flanking region, PXXSV<u>K</u>DR, are conserved in the plant CSases (Urano *et al.*, 2000). The CSaseLP amino acid

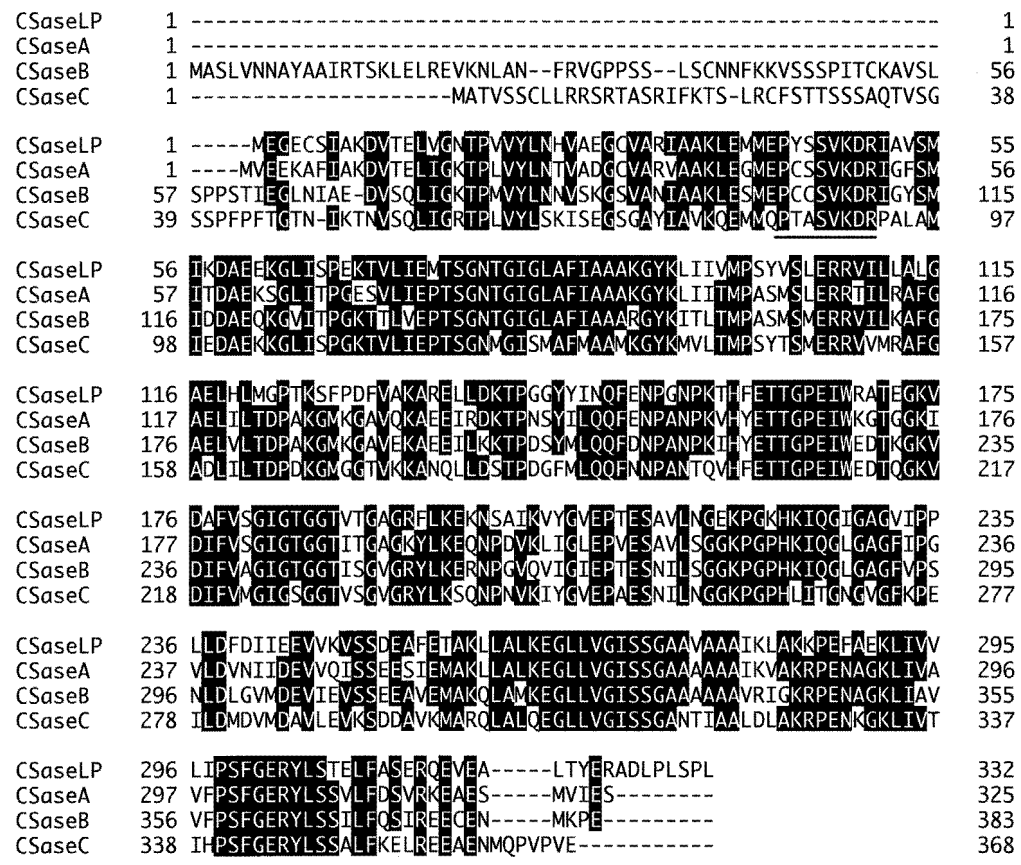

Fig. 1 Alignment of amino acid sequences deduced from the spinach genes encoding the CSaseLP, and cysteine synthases CSaseA, CSaseB and CSaseC. The underlined sequence indicates the pyridoxal phosphate binding motif conserved in plant CSase genes.

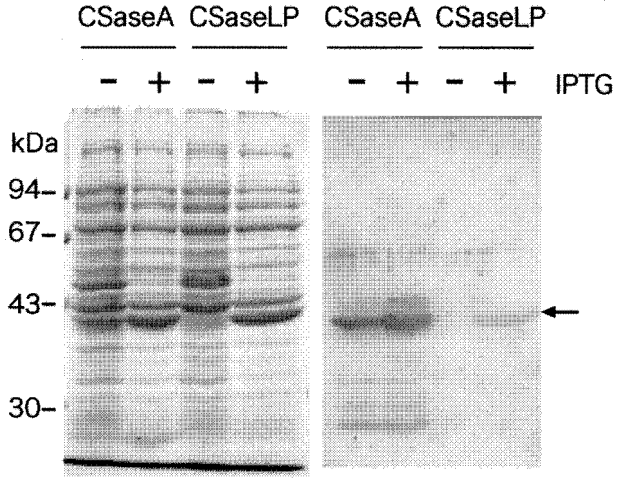

Fig. 2 Expression of the recombinant CSaseLP and CSaseA proteins in *E. coli*. Expression of recombinant proteins was induced by addition of IPTG. *E. coli* cells cultured without IPTG were also harvested. The left panel was the electrophoretic pattern on the SDS-PAGE. After electrophoresis, these proteins were subjected to the Western blot analysis with polyclonal anti-CSase antibody (right panel). An arrow indicates the position of recombinant proteins.

sequence also harbored this motif, suggesting that the CSaseLP protein is an isoform of the spinach CSase family.

The recombinant CSaseLP and CSaseA proteins with His tag were expressed in *E. coli* (**Fig. 2**). Most of the recombinant CSaseA protein was recovered in the soluble fraction, but the recombinant CSaseLP protein was expressed in an insoluble state. The recombinant CSaseLP protein was solubilized with 8 M urea, and then purified by metal-chelate affinity chromatography. Then urea was removed by dialysis under the presence of pyridoxal phosphate. The same protein folding procedure was applied to purify the recombinant CSaseA protein. The folded CSaseA protein exhibited CSase activity of 56.7 mmol min^{-1} (mg protein)$^{-1}$, but that of CSaseLP did not [0.0013 mmol min^{-1} (mg protein)$^{-1}$]. Small amounts of the recombinant CSaseLP protein were found in the soluble fraction prepared from the *E. coli* extracts, but the CSase activity in this soluble fraction was negligible (data not shown). As shown in **Fig. 2**, the recombinant CSaseLP protein reacted weakly with a rabbit antibody raised against spinach CSases (Kitamura *et al.*, 1996). When the recombinant CSaseLP and CSaseA proteins were subjected to the SDS-PAGE

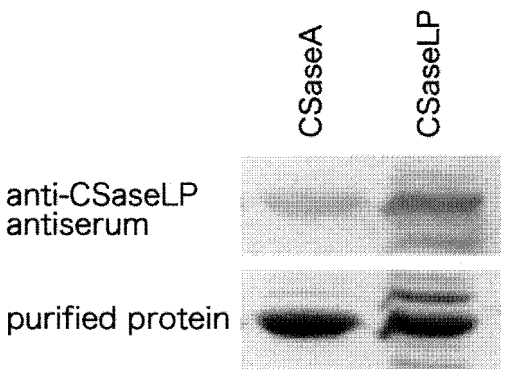

Fig. 3 Immunodetection of the recombinant CSaseLP and CSaseA proteins with an anti-CSaseLP antiserum. Each purified recombinant protein was separated on SDS-PAGE and then subjected to Western blot analysis. The upper panel was the immunostained recombinant proteins. Immunoblotting was carried out with rabbit polyclonal anti-CSaseLP antiserum. The lower panel is an electrophoretogram of the same proteins after staining with Coomassie brilliant blue.

and native PAGE, electrophoretic mobility of the recombinant CSaseLP protein was quite similar to that of the recombinant CSaseA protein (data not shown). This result implies that native CSaseLP protein exhibits similar electrophoretic mobility to that of native CSaseA protein. Then, we raised polyclonal rabbit antibody against the recombinant CSaseLP protein. The recombinant CSaseA protein cross-reacted weakly with the anti-CSaseLP antiserum (**Fig. 3**), suggesting that determination of *in vivo* protein level of the CSaseLP is difficult even if an anti-CSaseLP antibody is utilized. **Fig. 4** shows a Northern blot analysis of leaf and root total RNAs. The *CSaseA* gene was expressed in both leaf and root tissues, while the *CSaseLP* was expressed mainly in root tissues, but the amount of its mRNA in root tissues was significantly lower in comparison with that of *CSaseA* mRNA.

Here, a cDNA that encodes a CSaseLP was isolated from spinach leaves. The deduced amino acid sequence of the *CSaseLP* gene shows some characteristics to be seen in the plant CSase genes. The CSases belong to a family of β-substituted alanine synthases that require pyridoxal phosphate as a cofactor (Alexander *et al.*, 1994), and the cysteine synthase enzymes have an ability to catalyze multiple reactions, indicating that a functional assignment of CSase-like sequences merely on the base of sequence data alone is problematic (Jost *et al.*, 2000). Taken together, we concluded that the CSaseLP has an enzyme activity distinct from the CSase.

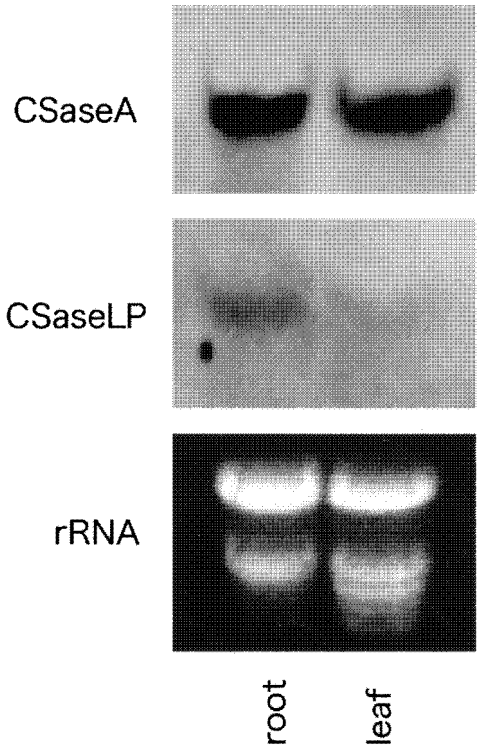

Fig. 4 Expression of the *CSaseLP* and *CSaseA* mRNA in leaf and root tissues. Total RNA (20 μg) extracted from leaf and root tissues was analyzed by Northern hybridization with the digoxygenin-labeled *CSaseLP* and *CSaseA* RNA probe. The equivalence of RNA loading among lanes was demonstrated by ethidium bromide staining of rRNA bands.

Reference

Alexander, F. W., Sandmeier, E., Mehta, P. K., Christen, P., 1994. Evolutionary relationships among pyridoxal 5′-phosphate-dependent enzymes. Eur. J. Biochem., **219**: 953-960.

Hatzfeld, Y., Maruyama, A., Schmidt, A., Noji, M., Ishizawa, K., Saito, K., 2000. β-Cyanoalanine synthase is a mitochondrial cysteine synthase-like protein in spinach and Arabidopsis. Plant Physiol., **123**: 1163-1171.

Jost, R., Berkowitz, O., Wirtz, M., Hopkins, L., Hawkesford, M. J., Hell, R., 2000. Genomic and functional characterization of the *oas* gene family encoding *O*-acetylserine(thiol)lyases, enzymes catalyzing the final step in cysteine biosynthesis in *Arabidopsis thaliana*. Gene, **253**: 237-247.

Kitamura, S., Yamaguchi, T., Zhu, X., Masada, M., 1996. Physiological study of cysteine synthase isozymes in *Spinacia oleracea* seedlings. Biosci. Biotech. Biochem., **60**: 1608-1611.

Kodama, H., Akagi, H., Kusumi, K., Fujimura, T., Iba, K., 1997. Structure, chromosomal location and expression of a rice gene encoding the microsome ω-3 fatty acid desaturase. Plant Mol. Biol., **33**: 493-502.

Maruyama, A., Ishizawa, K., Takagi, T., 2000. Purification

and characterization of β-cyanoalanine synthase and cysteine synthase from potato tubers: Are β-cyanoalanine synthase and mitochondrial cysteine synthase same enzyme? Plant Cell Physiol., **41**: 200-208.

Maruyama, A., Saito, K., Ishizawa, K., 2001. β-Cyanoalanine synthase and cysteine synthase from potato: molecular cloning, biochemical characterization, and spatial and hormonal regulation. Plant Mol. Biol., **46**: 749-760.

Murakoshi, I., Kaneko, M., Koide, C., Ikegami, F., 1986. Enzymatic synthesis of the neuroexcitatory amino acid quisqualic acid by cysteine synthase. Phytochemistry, **25**: 2759-2763.

Saito, K., Miura, N., Yamazaki, M., Hirano, H., Murakoshi, I., 1992. Molecular cloning and bacterial expression of cDNA encoding a plant cysteine synthase. Proc. Natl. Acad. Sci. U. S. A., **89**: 8078-8082.

Saito, K., Tatsuguchi, K., Murakoshi, I., Hirano, H., 1993. cDNA cloning and expression of cysteine synthase B localized in chloroplasts of *Spinacia oleracea*. FEBS Lett., **324**: 247-252.

Saito, K., Tatsuguchi, K., Takagi, Y., Murakoshi, I., 1994. Isolation and characterization of a cDNA that encodes a putative mitochondrion-localizing isoform of cysteine synthase (*O*-acetylserine(thiol)-lyase) from *Spinacia oleracea*. J. Biol. Chem., **269**: 28187-28192.

Urano, Y., Manabe, T., Noji, M., Saito, K., 2000. Molecular cloning and functional characterization of cDNAs encoding cysteine synthase and serine acetyltransferase that may be responsible for high cellular cysteine content in *Allium tuberosum*. Gene, **257**: 269-277.

Warrilow, A. G. S., Hawkesford, M. J., 2000. Cysteine synthase (*O*-acetylserine (thiol)lyase) substrate specificities classify the mitochondrial isoform as a cyanoalanine synthase. J. Exp. Bot., **51**: 985-993.

Yamaguchi, T., Masada, M., 1995. Comparative studies on cysteine synthase isozymes from spinach leaves. Biochim. Biophys. Acta, **1251**: 91-98.

Yamaguchi, T., Zhu, X., Masada, M., 1998. Purification and characterization of a novel cysteine synthase isozyme from spinach hydrated seeds. Biosci. Biotech. Biochem., **62**: 501-507.

Yamaguchi, Y., Nakamura, T., Kusano, T., Sano, H., 2000. Three *Arabidopsis* genes encoding proteins with differential activities for cysteine synthase and β-cyanoalanine synthase. Plant Cell Physiol., **41**: 465-476.